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13. ABSTRACT (Maximum 200 Words) This grant proposes to determine whether organophosphate insecticides act upon the cholinergic system in the lungs increasing cholinergic neurotransmission and causing airway hyperresponsiveness, which is characteristic of asthma. Guinea pigs were either treated acutely with a high dose or chronically (7 days) with a low dose of the organophosphate, chlorpyrifos sc. Electrical stimulation of the vagus nerves caused frequency-dependent bronchoconstriction that was significantly potentiated in animals treated with chlorpyrifos. M2 muscarinic autoreceptors, which normally inhibit release of acetylcholine from cholinergic nerves were dysfunctional in the chlorpyrifos -treated animals. The function of M3 muscarinic receptors on airway smooth muscle was not altered by chlorpyrifos treatment. In addition, the high but not the lower dose of chlorpyrifos significantly inhibited acetylcholinesterase activity, further contributing to airway hyperreactivity. These data demonstrate that organophosphate pesticides are capable of causing airway hyperreactivity by inhibiting neuronal M2 receptor function. A second series of experiments has demonstrated that 2 additional organophosphates, diazinon and paration also inhibit M2 receptor function and cause airway hyperreactivity, experiments to determine the lowest level of exposure are underway. Thus, we have confirmed that organophosphates cause airway hyperactivity by inhibiting M2 receptor function on the cholinergic nerves and by inhibiting acetylcholinesterase activity.		
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INTRODUCTION

Over the past 20 years there has been a significant increase in the incidence of asthma in industrialized nations. Over the same timeframe, insecticide usage has increased significantly, not only in agricultural settings, but also in the inner cities. One of the most commonly used classes of insecticides is the organophosphates and a number of clinical reports and epidemiological studies have linked exposure to organophosphates to airway hyperreactivity and asthma. However, the mechanism by which these insecticides cause changes in airway function remain unknown. In the lung, cholinergic nerves in the vagi mediate airway tone and reactivity. These nerves release acetylcholine onto M3 muscarinic receptors causing contraction of airway smooth muscle resulting in bronchoconstriction. Vagally induced bronchoconstriction is limited by autoinhibitory M2 muscarinic receptors on parasympathetic nerves. We have shown that previously that neuronal M2 receptors are dysfunctional in animal models of asthma. Loss of M2 receptor function leads to increased release of acetylcholine from the parasympathetic nerves resulting in potentiation of vagally-mediated bronchoconstriction, which contributes to airway hyperreactivity. Since organophosphates are known to alter cholinergic function in the brain, we tested whether organophosphate insecticides can alter neuronal M2 muscarinic receptor function in the lungs and induce hyperreactivity.

BODY:

There are 3 specific aims:

1. To determine whether organophosphates cause airway hyperreactivity.
2. To determine the mechanism of action by which the organophosphates induced hyperactivity
3. To determine whether exposure to organophosphates exacerbated airway hyperreactivity following antigen challenge.

Results: (All figures are attached at the end of this report in the appendix) Treatment with the organophosphate chlorpyrifos did not result in any apparent signs of cholinergic intoxication 1 or 24 hr following the injections. Neither chlorpyrifos nor peanut oil altered baseline pulmonary

inflation pressure (control 91 ± 7 mmH₂O, peanut oil 96 ± 5 mmH₂O, 70 mg/kg chlorpyrifos 87 ± 7 mmH₂O, 390mg/kg chlorpyrifos 96 ± 5 mmH₂O), resting heart rate (control 281 ± 11 beats/min, peanut oil 271 ± 8 beats/min, 70 mg/kg chlorpyrifos 268 ± 9 beats/min, 390mg/kg chlorpyrifos 300 ± 7 beats/min) or resting blood pressure (diastolic: control 45 ± 2.3 mmHg, peanut oil 41 ± 2 mmHg, 70 mg/kg chlorpyrifos 46 ± 2 mmHg, 390mg/kg chlorpyrifos 56 ± 2.4 mmHg) in vagotomized, anesthetized guinea pigs.

Electrical stimulation of both vagi (1-25 Hz) caused a frequency dependent increase in bronchoconstriction that was significantly potentiated in animals treated with chlorpyrifos whether they received 390mg/kg for 24 hours or 70mg/kg for 7 days (Figure 1). However, a greater increase in bronchoconstriction was observed in animals that received the acute high dose chlorpyrifos treatment. Vagally induced bronchoconstriction was not altered in animals receiving vehicle alone (peanut oil) relative to controls.

Neuronal M2 receptor function was tested in chlorpyrifos-treated animals with the muscarinic agonist pilocarpine. Prior to administering pilocarpine, simultaneous electrical stimulation of both vagus nerves (2Hz, 0.2ms, 5-20Volts, 22 sec at 1-minute intervals) produced transient bronchoconstriction (measured as an increase in Ppi) that did not differ between groups (control 27.6 ± 0.2 mmH₂O, peanut oil 19.8 ± 3 mmH₂O, 70 mg/kg chlorpyrifos 18.6 ± 5 mmH₂O, 390mg/kg chlorpyrifos 24.6 ± 6 mmH₂O). In guinea pigs treated with peanut oil, pilocarpine (1-100 μ g/kg, iv) dose-dependently inhibited vagally induced bronchoconstriction (Figure 2, open circles). The effect was identical to saline treated controls (not shown), demonstrating that injection of peanut oil sc for 7 days did not alter the function of neuronal M2 receptors. The dose response curve to pilocarpine was shifted significantly to the right in animals treated with chlorpyrifos at 390mg/kg for 24 hours. A lesser, but still significant rightward shift was observed following 70mg/kg chlorpyrifos treatment for 7 days.

The direct response of airway smooth muscle to muscarinic agonists was tested by measuring bronchoconstriction induced by intravenous methacholine and acetylcholine in vagotomized guinea pigs. Chlorpyrifos treatment did not alter bronchoconstriction induced by methacholine (Figure 3A). In contrast, acetylcholine induced bronchoconstriction was significantly increased in the 390mg/kg chlorpyrifos for 24 hour animals but not in the animals treated with 70 mg/kg for 7 days (Fig. 3B).

In the heart, stimulation of the vagus nerves (1-25 Hz) produces bradycardia that is frequency dependent (Figure 4). Treatment with 70mg/kg chlorpyrifos for 7 days did not alter the vagally induced fall in heart rate relative to control. However, in the animals treated with 390mg/kg chlorpyrifos for 24 hours, the frequency response curve was potentiated at frequencies less than 20Hz (Fig. 4). At 20 and 25 Hz, the fall in heart rate was approaching maximum and the differences are no longer significant. Methacholine- and acetylcholine-induced bradycardia was not altered by either chlorpyrifos treatment (Figure 5).

The effect of chlorpyrifos on AChE was also measured. Chlorpyrifos treatment at 70mg/kg for 7 days had no effect on lung AChE. However acute treatment with 390mg/kg chlorpyrifos for 24 hour inhibited lung AChE by 50% and blood AChE by nearly 90% (Figure 6).

Key Research Accomplishments:

- 1: One organophosphate, chlorpyrifos, causes airway hyperreactivity to electrical stimulation of the vagi.
- 2: Chlorpyrifos does not cause airway hyperractivity to intravenous methacholine, a muscarinic agonist not metabolized by acetylcholinesterase.
3. The function of inhibitory M2 receptors on the parasympathetic nerves is inhibited by chlorpyrifos, this would increase release of acetylcholine and may be one of the mechanisms of chlorpyrifos induced hyperreactivity.
4. Chlorpyrifos at high doses will inhibit acetylcholinesterase. This is reflected in increased bronchoconstriction to iv acetylcholine in the lungs and is another mechanism for airway hyperreactivity.

Reportable outcomes:

At this point we are writing a manuscript containing the data in figures 1-6

Conclusions:

Airway hyperreactivity was measured in guinea pigs 24 hr after injection with 390 mg/kg or 7 days after injection with 70 mg/kg of chlorpyrifos sc. Electrical stimulation of the vagus nerves caused a frequency-dependent bronchoconstriction that was significantly potentiated in animals treated with chlorpyrifos. Neuronal M2 receptor function was tested with pilocarpine, which inhibits vagally-induced bronchoconstriction in control animals. In animals treated with chlorpyrifos, the pilocarpine dose-response curve was shifted significantly to the right, demonstrating decreased responsiveness of the neuronal M2 receptors. The function of M3 muscarinic receptors on airway smooth muscle was not altered by chlorpyrifos treatment. In addition, 390 but not 70 mg/kg of chlorpyrifos significantly inhibited acetylcholinesterase activity, further contributing to airway hyperreactivity. These data demonstrate that organophosphate insecticides are capable of causing airway hyperreactivity by inhibiting neuronal M2 receptor function.

Thus we have completed aim 1 and 2 for one of the organophosphates. We are currently undertaking similar experiments examining the organophosphates diazinon and parathion..



A handwritten signature in black ink, appearing to read "P.I." followed by a stylized surname.

Vagally induced bronchoconstriction is increased by chlorpyrifos in guinea pigs.

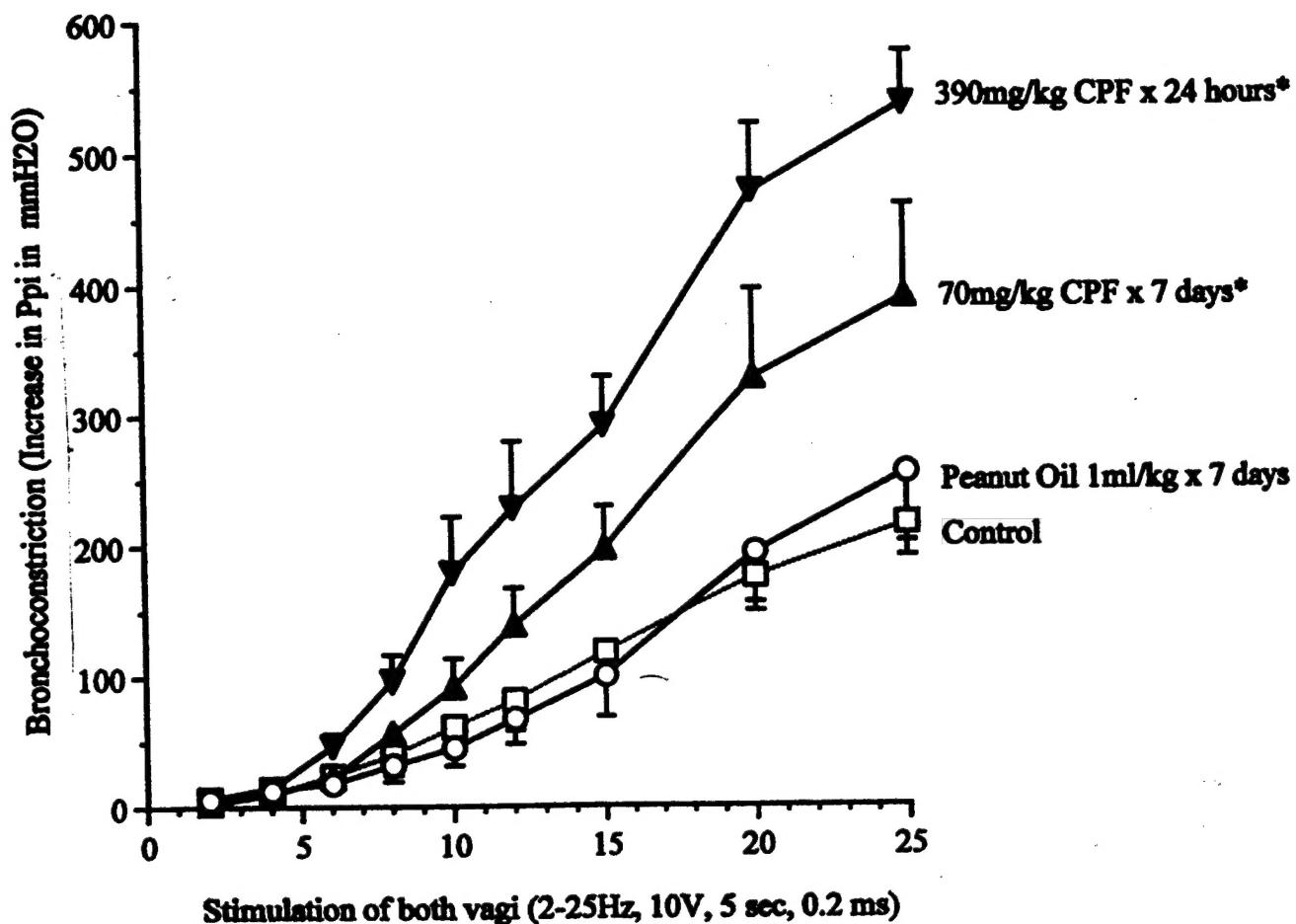


Fig. 1. Electrical stimulation of the vagus nerves (1-25 Hz, 10V, 0.2ms, 5 second train) produced frequency dependent bronchoconstriction in the lungs, measured as an increase in pulmonary inflation pressure (open squares). Vagally induced bronchoconstriction was significantly increased in animals treated with 70mg/kg chlorpyrifos for 7 days (filled upward triangles) and with 390mg/kg chlorpyrifos for 24 hours (filled downward triangles). Frequency response curves in animals treated with peanut oil vehicle for 7 days (open circles) were not different from control animals. Each point is the mean +/-SEM of 5-8 animals; *significantly different from control.

**Chlorpyrifos inhibits neuronal M₂ muscarinic receptors
in a dose related manner.**

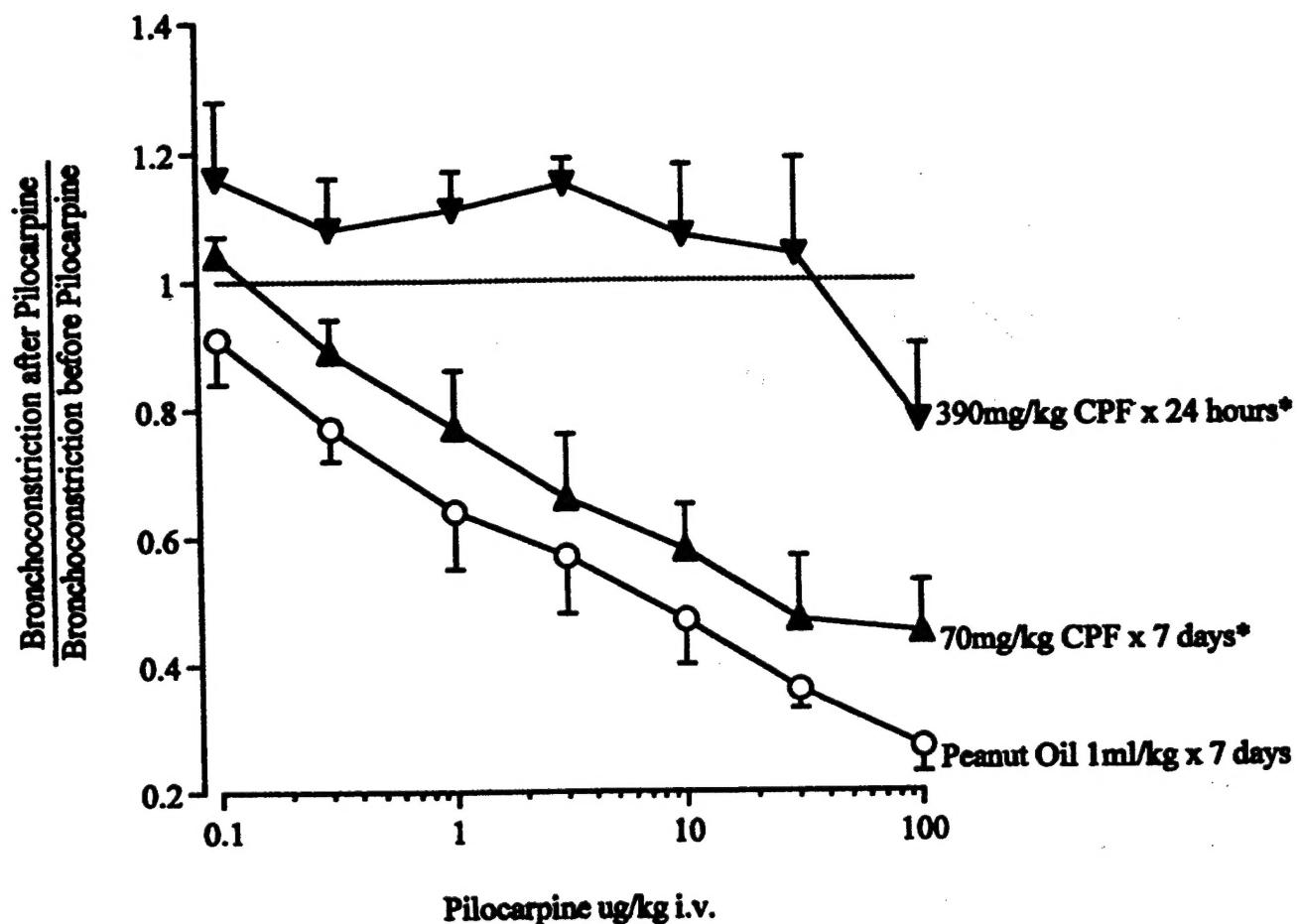
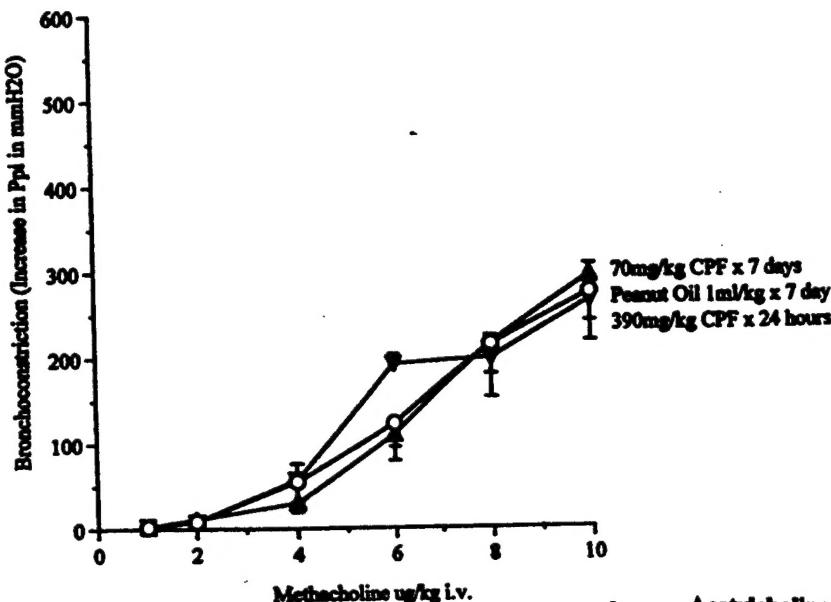


Fig. 2. Neuronal M₂ receptor function was tested using pilocarpine. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals treated with peanut oil for 7 days (open circles) demonstrating functional M₂ receptors. The effect of pilocarpine was shifted significantly to the right in animals treated with 70mg/kg chlorpyrifos for 7 days (filled upward triangles). In the animals treated with 390mg/kg chlorpyrifos for 24 hours (filled downward triangles) pilocarpine did not inhibit vagally induced bronchoconstriction indicating neuronal M₂ muscarinic receptor dysfunction. Each point is the mean +/- SEM of 5 animals; *significantly different from control.

A. Methacholine-induced bronchoconstriction is not increased by chlorpyrifos in guinea pigs



B. Acetylcholine-induced bronchoconstriction is only increased by a high dose of chlorpyrifos in guinea pigs.

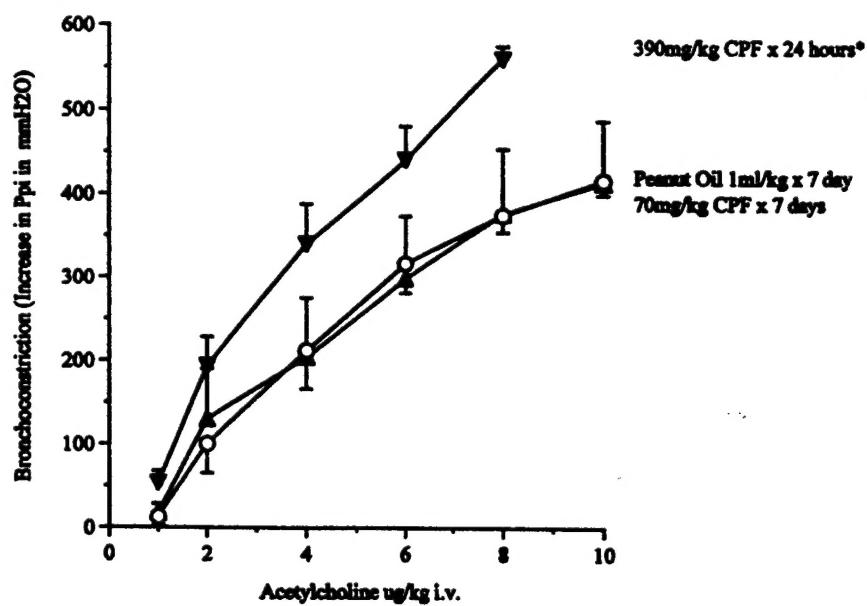


Fig. 3. (A) Methacholine (1-10 μ g/kg, iv) induced bronchoconstriction in vagotomized guinea pigs was not different between peanut oil (open circles) or chlorpyrifos treated guinea pigs (70mg/kg chlorpyrifos for 7 days; filled upward triangles and 390mg/kg chlorpyrifos for 24 hours; filled downward triangles). (B) Acetylcholine (1-10 μ g/kg, iv) induced bronchoconstriction in vagotomized guinea pigs was significantly potentiated by 390 mg/kg chlorpyrifos. Each point is the mean +/- SEM of 5 animals
*significantly different from oil control.

Vagally induced bradycardia is increased by chlorpyrifos in guinea pigs.

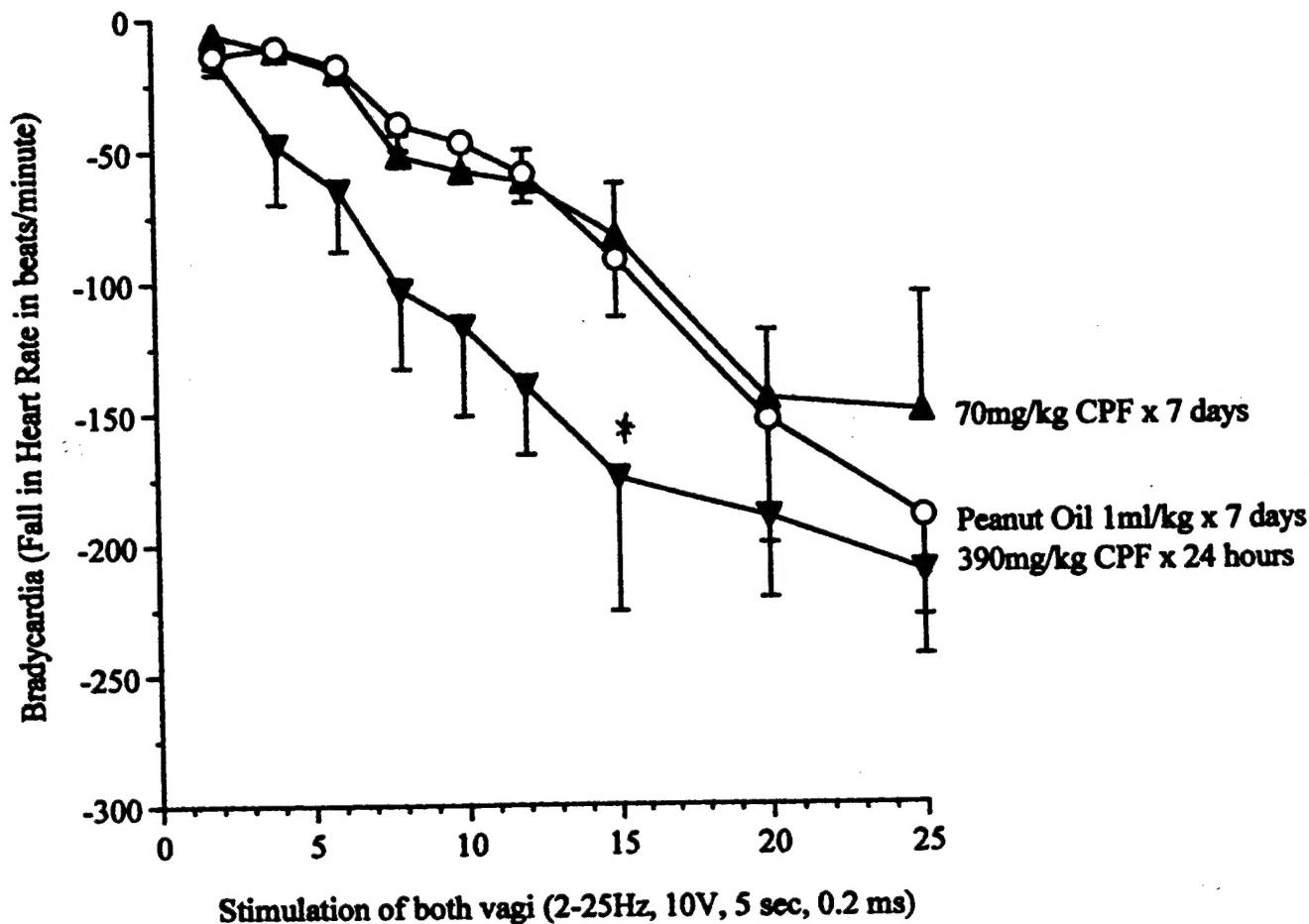
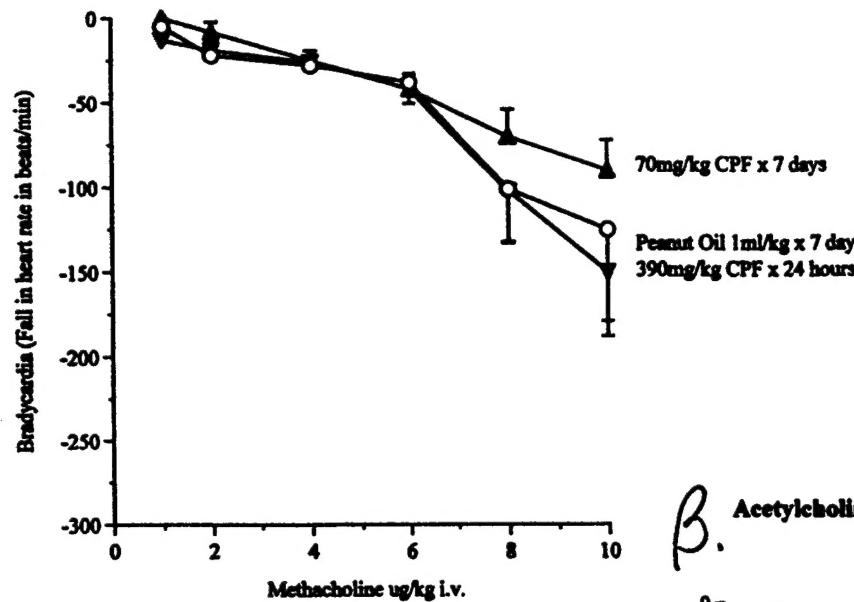


Fig. 4. Electrical stimulation of the vagus nerves (1-25 Hz, 10V, 0.2ms, 5 second train) produced frequency dependent bradycardia in the heart measured as a fall in heart rate (open circles). Vagally induced bradycardia in animals treated with 70mg/kg chlorpyrifos for 7 days (filled upward triangles) was not significantly different from peanut oil treated animals. However, vagally induced bradycardia in animals treated with 390mg/kg chlorpyrifos for 24 hours (filled downward triangles) was shifted the left. Up until 20Hz, the shift was significantly different from oil control, however, at 20 and 25 Hz, the fall in heart rate was approaching maximum and the differences are no longer significant. Each point is the mean +/-SEM of 5-8 animals; *significantly different from control.

A.

Methacholine-induced bradycardia is not altered by chlorpyrifos in guinea pigs.



B.

Acetylcholine-induced bradycardia is not altered by chlorpyrifos in guinea pigs.

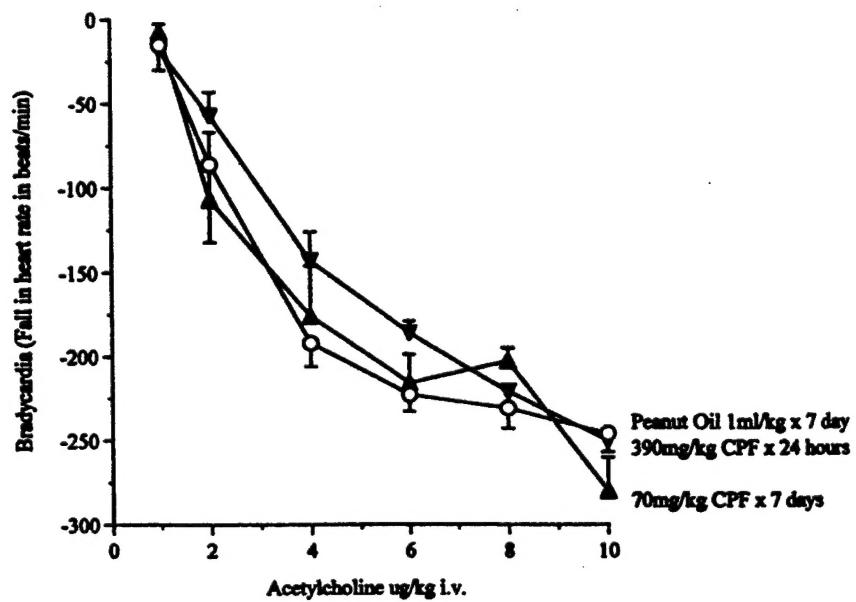


Fig. 5. (A) Methacholine (1-10 μ g/kg, iv) -induced bradycardia in vagotomized guinea pigs was not different between peanut oil (open circles) or chlorpyrifos treated guinea pigs (70mg/kg chlorpyrifos for 7 days; filled upward triangles and 390mg/kg chlorpyrifos for 24 hours; filled downward triangles). **(B)** Acetylcholine (1-10 μ g/kg, iv) -induced bradycardia in vagotomized guinea pigs was not different between peanut oil (open circles) or chlorpyrifos treated guinea pigs (70mg/kg chlorpyrifos for 7 days; filled upward triangles and 390mg/kg chlorpyrifos for 24 hours; filled downward triangles). Each point is the mean \pm SEM of 5 animals.

**Chlorpyrifos (390mg/kg sc)
inhibits AChesterase in Guinea Pigs**

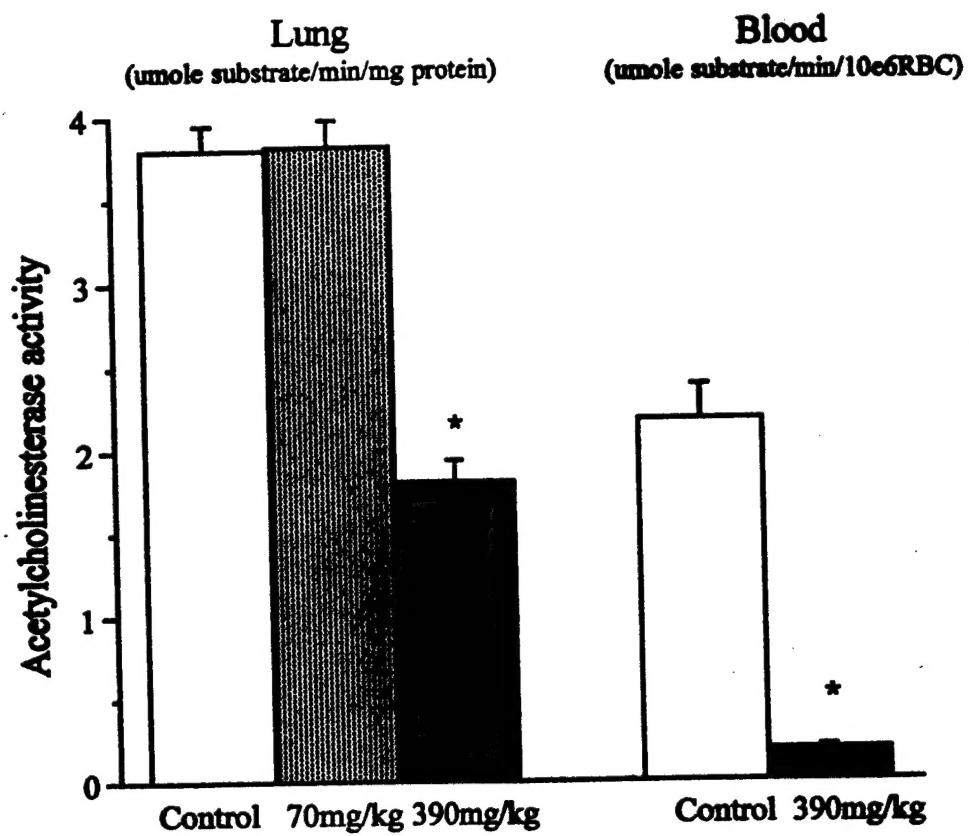


Fig. 6. Acetylcholinesterase activity in the lungs of animals treated with 70mg/kg chlorpyrifos for 7 days (shaded bars) was not different from control (open bars). In contrast, 390mg/kg chlorpyrifos for 24 hours (filled bars) significantly inhibited acetylcholinesterase activity in the lungs and in the blood. Each point is the mean +/- SEM of 5-8 animals. *significantly different from control.